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RAPID ATTENUATION OF VEE VIRUS IN CHRONICALLY INFECTED SUSPENDED L CELLS

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ABSTRACT

L cells grown in serum-free defined medium readily became chronically infected with Venezuelan equine encephalomyelitis (VEE) virus whereas cells grown in serum-free lactalbumin medium did not. The addition of serum eliminated the ability of the defined medium to induce the chronic state of infection. During the first 48 hours after inoculating L cell cultures, the cells underwent an acute phase of viral activity that resulted in widespread cell destruction and 10^8 to 10^9 $MICLD_{50}$ of virus per ml. The acute phase was followed by an apparent transitional phase with yields of approximately 10^4 $MICLD_{50}$ of virus per ml, then a resumption of cell multiplication. As the cultures progressed into the chronically infected phase, virus yields fluctuated between 10^3 and $10^{5.5}$ $MICLD_{50}$ per ml. Finally, the virus lost its ability to produce a lethal intraperitoneal infection at 8 days for rabbits, at 29 days for mice, at 72 days for guinea pigs, and at 112 days for hamsters. The qualitative changes in the viral populations that were derived from the culture occurred, therefore, independently of concomitant quantitative changes. During the course of chronic infection, the cells were resistant to a challenge with homotypic virus.

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RAPID ATTENUATION OF VEE VIRUS IN CHRONICALLY INFECTED SUSPENDED L CELLS

We previously reported[1] that L cell monolayer cultures, chronically infected with Venezuelan equine encephalomyelitis (VEE) virus, eventually yielded viral progeny that were markedly different from the original parent population. One of the most significant characteristics shown by the new virus was a loss of virulence that was demonstrable in a variety of laboratory animals. Although the mechanism for the selection of the nonlethal variant population in chronically infected L cell monolayer cultures has not been elucidated, it was recognized that the average length of time in continual residence in cell culture that was necessary for the conversion to avirulence was 7 to 9 months.

Evidence that suspensions of L cells grown in serum-free media would support the growth of VEE virus led to the additional disclosure that cells grown in this manner could also become chronically infected with VEE virus. This paper reports some of the biological events that are associated with this phenomenon.

The parent VEE virus inoculum used to infect the L cells was an embryonated egg preparation that was highly virulent for mice,* guinea pigs, hamsters, and rabbits by the intraperitoneal (ip) route. The cell lines employed were grown in 30-ml amounts in 100-ml medium bottles in a New Brunswick Gyrotory shaker at 35 C. The media used during the tests were a lactalbumin hydrolyzate medium and a chemically defined medium containing either essential or nonessential amino acids. These media have been described by Higuchi[2] and by Nagle, Tribble, Anderson and Gary,[3] respectively. Cultures were inoculated in the following manner: Virus was added to media prior to the addition of cells, and samples were obtained to ascertain the initial viral concentration. Cells were added to make a final concentration of 1.5×10^6 cells per ml and the virus-cell mixture was incubated for 45 minutes on a shaker. This culture was then centrifuged and the cells were washed once in fresh medium and resuspended. At that time a 0-time virus sample was obtained; additional samples were taken at various intervals to be titrated in 12- to 14-gram mice. Virus titers are expressed as mouse intracerebral (MIC) and mouse intraperitoneal (MIP) LD_{50} per ml of culture fluid. Animals that did not succumb as a result of the virus infection were challenged 21 days later with a lethal dose of VEE virus to determine whether they had developed an immunity even though they had shown no clinical signs of infection.

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* In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

The results of tests that were performed by infecting cultures grown in defined or in lactalbumin medium disclosed that only in the defined medium were the chronically infected L cell cultures effectively established with VEE virus. Media that failed to produce this effect include the lactalbumin medium, lactalbumin supplemented with 10% serum, and defined medium supplemented with 10% serum.

Figure 1 shows typical experimental results comparing the effect of the lactalbumin and defined media on viral yields. The upper curve shows that with the lactalbumin medium, maximal viral yields of 10^9 $MICLD_{50}$ per ml were obtained at 24 to 48 hours. Thereafter, the viral titer steadily declined so that by day 8 approximately $10^{1.5}$ $MICLD_{50}$ per ml of virus remained. During that time, cells in the culture lysed so that shortly after day 4, few if any cells remained in the culture.

In contrast maximal viral proliferation of approximately 10^8 $MICLD_{50}$ per ml was obtained in defined medium at 24 to 48 hours as shown by the middle curve on the figure. This proliferation decreased to approximately 10^4 $MICLD_{50}$ per ml on day 4. This, or slightly higher titers persisted, however, for 35 days in marked contrast to the previous case with the lactalbumin medium in which little or no virus was found beyond day 4. The cell population in the defined medium culture declined on Days 1 and 2 from 10^8 cells per ml to approximately 10^6 cells and remained at that level until cellular multiplication resumed after day 7.

The lowest curve on Figure 1 represents a titration of virus in mice by the ip route, which was used concomitantly with the previously mentioned ic method of titrating virus grown in defined medium. At day 1, titration by the ip method was tenfold less sensitive than when the ic route was used. Between day 4 and day 7, however, a far greater difference was observed. During that time, it became increasingly difficult to detect virus by the ip titration method even though substantial amounts of virus could be demonstrated by the ic method. Therefore, one week in suspended culture appeared to be sufficient time to produce viral attenuation that previously required 7 to 9 months in L cell monolayer cultures.

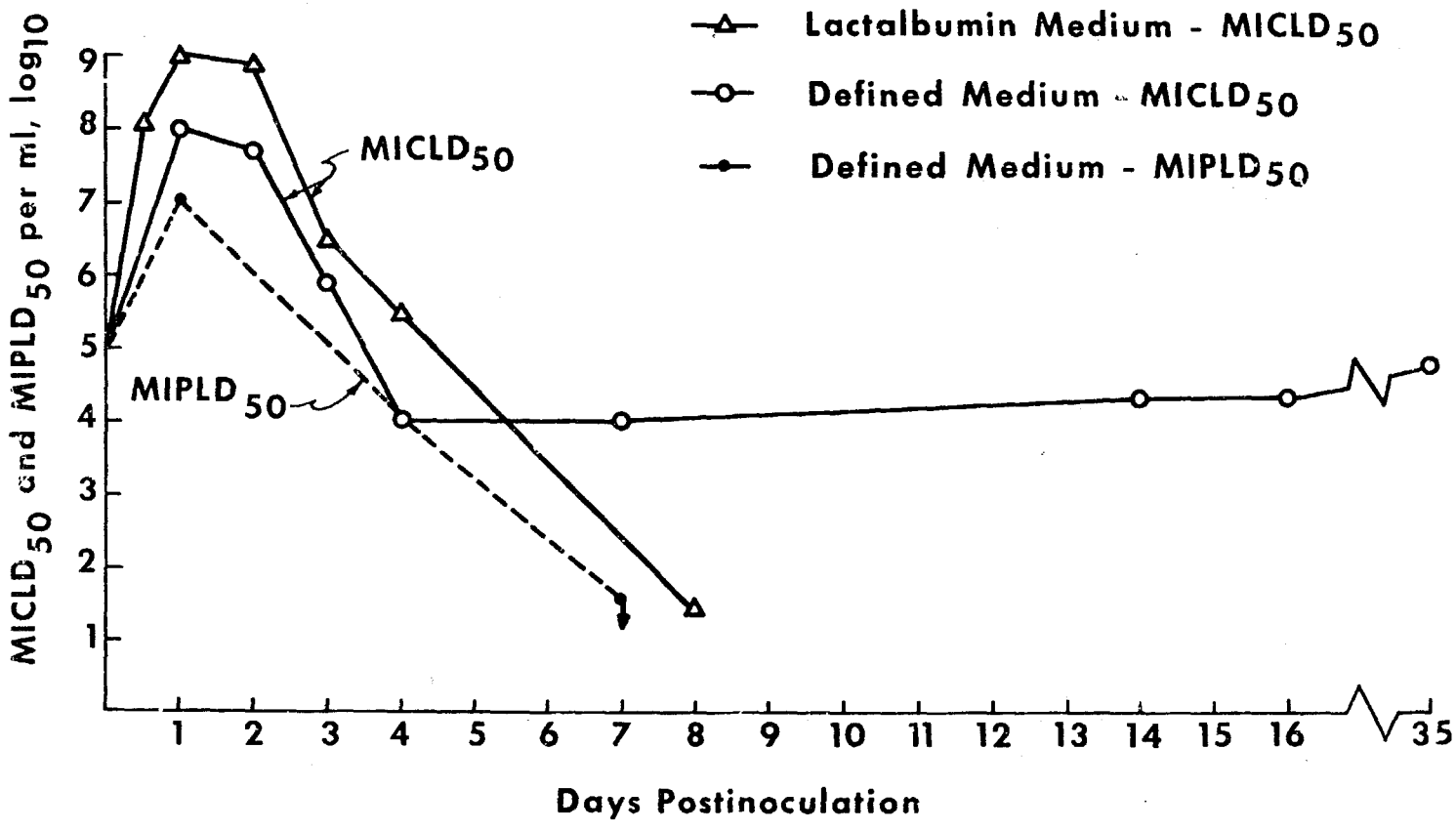

Figure 1. VEE Virus Replication in L Cells Suspended in Defined or Lactalbumin Medium.

The rapidity at which this phenomenon occurred in suspension cultures prompted further investigations. Chronically infected cultures were established to study more closely the viral growth that occurred during periods longer than 35 days. For the first 4 days, a typical response to viral infection was found. Maximal titers of $10^{8.2}$ $MICLD_{50}$ per ml occurred at day 2 and were associated with $MIPLD_{50}$ titers that were between 10- and 100-fold less. The data in Table 1 represent the unstable and transient character of the virus between days 5 and 25. For example, on day 5, $10^{3.0}$ $MICLD_{50}$ per ml of virus was present. The virus obtained at this time was not lethal for mice by the ip route. On days 6 and 8, however, some ip-lethal virus recurred; it disappeared on days 10 and 12 but was found again on day 14 and, next, on day 24. During the following interval however, the viral population appeared to stabilize. Virus that was lethal for mice by the ip route was not recovered again even though the culture continued to produce approximately $10^{4.5}$ $MICLD_{50}$ of virus. The appearance and disappearance of ip lethal virus, therefore, did not appear to reflect any changes in the overall concentration of virus as determined by the ic method of assay.

TABLE 1. VIRUS REPLICATION IN SUSPENDED L CELL CULTURES CHRONICALLY INFECTED WITH VEE VIRUS

Day Postinoculation	Viral Titer, $\log_{10}$ per ml	
	$MICLD_{50}$	$MIPLD_{50}$
4	4.0	2.8
5	3.0	<1.6[a]
6	3.0	2.8
8	4.8	2.4
10	4.2	<1.6
12	4.2	<1.6
14	4.0	2.5
18	4.0	<1.6
24	4.7	2.5
29	4.8	<1.6
35	5.6	<1.6
70	4.9	<1.6
112	5.5	<1.6

a. No virus detected in 1:10 dilutions of virus.

Additional tests disclosed that other qualitative changes that seemed independent of any concomitant quantitative changes occurred in the virus population.

Inasmuch as the attenuated VEE virus strain that was mentioned in the introduction is nonlethal for common laboratory animals, virus obtained from the chronically infected cultures was tested additionally in rabbits, hamsters, and guinea pigs. The results given in Table 2 show that by day 8 virus that was recovered from the culture was not lethal for rabbits but retained its lethality for mice, guinea pigs, and hamsters. After 24 days, the virus was no longer lethal for mice, at 72 days it was not lethal for guinea pigs, and at 112 days it was not lethal for hamsters. The loss of lethality for the various animal species, however, was accompanied by an immunogenic response. All animals that did not give a lethal response were found to be resistant to a lethal dose of the homotypic parent virus.

TABLE 2. LETHALITY OF VEE VIRUS FROM CHRONICALLY INFECTED L CELL SUSPENDED CULTURES

Animal	Age of Culture, days				
	5	8	24	72	112
Rabbits	±[a]	-	-	-	-
Mice	+	+	-	-	-
Guinea Pigs	+	+	+	-	-
Hamsters	+	+	+	+	-

a. + Lethal, - Nonlethal.

Finally one of the classical characteristics of chronically infected cultures was demonstrated. At 70 days, the culture was challenged with a virulent strain of VEE virus. As indicated earlier, the presence of virus of this type could easily be detected by its lethality when injected intraperitoneally into mice. No evidence of replication of ip-virulent virus was found thereby indicating the resistance of the culture to superinfection with a homotypic virus. The culture was not resistant to superinfection with a rickettsial organism.

In summary, biological factors associated with the establishment of suspended L cell cultures chronically infected with Venezuelan equine encephalomyelitis (VEE) virus were presented. Cells grown in a serum-free defined medium readily became chronically infected with VEE virus, whereas cells grown in serum-free lactalbumin medium did not. The addition of serum eliminated the ability of the defined medium to induce the chronic state of infection. Prior to the establishment of chronicity in the cultures, the cells underwent an acute phase of viral activity during the first 48 hours postinfection. This was characterized by the presence of 10^8 to 10^9 $MICLD_{50}$ of virus per ml and widespread cell destruction, both of which diminished at day 4. The acute phase was followed by an apparent transitional phase with yields of approximately 10^4 $MICLD_{50}$ of virus per ml and a resumption of cell multiplication. Based upon the infectivity of this virus in mice, the transitional phase appeared to last approximately 3 weeks. During that time, the viral population appeared to be relatively unstable as evidenced by the cyclic recurrence of small quantities of virus that could produce a lethal infection by the ip route in mice. As the cultures progressed into the chronically infected phase, virus yields that fluctuated between 10^3 and $10^{5.5}$ $MICLD_{50}$ per ml were found. In a step-wise manner such virus rapidly lost its ability to produce lethal infections in common laboratory animals; i.e., virus was no longer lethal by the ip route at 8 days for rabbits, at 29 days for mice, at 72 days for guinea pigs, and at 112 days for hamsters. The qualitative changes in the viral populations that were derived from the culture, occurred, therefore, independently of concomitant quantitative changes. During the course of chronic infection, the cells were resistant to a challenge with virulent VEE virus.

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